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PRINCIPAL INVESTIGATOR: Anindya Dutta, M.D., Ph.D.

CONTRACTING ORGANIZATION: Brigham and Women's Hospital
Boston, Massachusetts 02115

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6. AUTHOR(S)
Anindya Dutta, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Brigham and Women's Hospital
Boston, Massachusetts 02115

E-MAIL:
adutta@rics.bwh.harvard.edu

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13. ABSTRACT *(Maximum 200 Words)*

Cy or RXL motifs have been previously shown to be cyclin binding motifs found in a wide range of cyclin-cdk interacting proteins. For both cyclin A/cdk2 and cyclin E/cdk2, the presence of a Cy motif decreased the $Km^{peptide}$ 75 to 120-fold while the kcat remained unchanged. Changes in the length of the linker between the Cy motif and the phosphoacceptor serine suggest that both sites are engaged simultaneously to the cyclin and the cdk, respectively. PS100, a peptide containing a Cy motif, acts as a competitive inhibitor of cyclin/cdk complexes with a 15-fold lower Ki for cyclin E/cdk2 than for cyclin A/cdk2. These results provide kinetic proof that a Cy motif located at a minimal distance from the SPXK is essential for optimal phosphorylation by cdks and suggest that small chemicals that mimic the Cy motif would be specific inhibitors of substrate recognition by cyclin-dependent kinases. In addition, a detailed mutagenesis approach to define what sequence serves as a Cy motif reveals that RXL is neither necessary nor sufficient to act as a Cy motif. Instead, the results suggest that a hydrophobic molecule that spans the length of 3-4 peptide bonds would be a suitable Cy-mimetic inhibitor of cdk.

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INTRODUCTION:

In the introduction to the annual report of '98 we discussed the importance of p53 function in breast cancer and how p53 exerts part of its tumor suppressor activity through the induction of p21. In the annual report of '99 we discussed the discovery of the cyclin binding Cy motifs on the p21 molecule and the realization that similar Cy motifs are present of substrates of cyclin-dependent kinases. We will briefly re-visit the latter points here.

Cy motif on inhibitors of cdks: The Cy motif was conserved in well known inhibitors of cdks: p21, p27 and p57. Together with our biochemical data, the crystallographic structure of cyclin A-cdk2 complexed with p27 (9) suggests that the Cy motif- cyclin interaction serves as a docking interaction essential for the complete interaction between cdk inhibitors and cyclin-cdk.

Cy motif on substrates of cdks: Cyclin A/cdk2 and cyclin E/cdk2 both play a major role in the G_1/S transition of the cell cycle by the phosphorylation of various substrates including pRB, E2F, and CDC6. Despite their critical role in this process, little is known about how these substrates are targeted to specific cyclin-cdk complexes.

Since the S/T-P-X-K/R consensus phosphorylation site is broadly applicable to all substrates of all cdks, it would be incapable of conferring the substrate specificity seen within a cellular context. An alternate mechanism by which this specificity could be achieved is through the presence of a docking site on the substrate that recruits the appropriate cyclin-cdk to the protein.

The crystal structure of the cdk inhibitor p27 complexed with cyclin A/cdk2 (9) indicates that the N-terminal Cy motif of p27 is bound to a shallow hydrophobic groove on the surface of the cyclin. Although there is no structural evidence to confirm it, it seems likely that substrates containing a Cy motif would bind in a similar fashion as the inhibitor. The Cy motif of the substrate would bind to the same groove on the cyclin and allow potential phosphorylation sites on the protein to associate with the nearby cdk2 subunit and become phosphorylated.

CDC6, a substrate of cdk2 that is dependent on its Cy motif for phosphorylation by cdk2: One substrate which we propose acts in this fashion is the human replication factor, CDC6. This factor is involved in the formation of a pre-replication complex and is required for the initiation of DNA replication. At the onset of S-phase, mammalian CDC6 is phosphorylated by cyclin A/cdk2which inactivates it by exporting it from the nucleus into the cytoplasm (7,12). Phosphopeptide analysis has shown that this phosphorylation by cyclin A/cdk2 occurs on Ser-54, Ser-74, and Ser-106 (13). This requires the presence of a nearby Cy motif at residues 94-98 as evidenced by the fact that point mutation of this sequence abolishes phosphorylation at these sites (Delmolino and Dutta, unpublished results).

Kinetic analysis of the contribution of a Cy motif is done more quantitatively if the Cy motif is on a substrate (like CDC6) than on an inhibitor (like p21): Although the kinetic analysis was originally written to study how the Cy motif contributes to the inhibition of cdks by p21, the availability of a substrate that utilizes a similar Cy motif made the analysis much easier. Last year we reported preliminary data on the phosphorylation of substrates with and without Cy motifs and suggested that the Cy motif make a 500-1000 fold to the Km of the substrate. The peptide without the Cy motif had a deletion in the Cy sequence. Therefore, one of the tasks completed this year was to make a comparison with a substrate peptide of the same length as the wild type peptide but carrying point mutations that inactivate the Cy motif rather than a deletion.

BODY

Statement of Work:

Technical objective 1. Analysis of the interaction between p21, cyclin and cdk.

Task 1: Months 1-24: Oligonucleotide directed mutagenesis of the portion of p21 in the cyclin binding site to determine what sequence feature is essential for binding to cyclins.
Completed

Task 1: Months 1-24: Lineweaver-Burke analysis of the inhibition of cyclin E-cdk2 by intact p21 and by p21 without the cyclin binding site.
Completed

Task 2: Months 24-36: Determination of the binding affinity of the cyclin-binding site for cyclins and of the cdk2 binding site for cdk2 and comparison with the binding affinity of intact p21 with the cyclin E-cdk2 holoenzyme.
Completed.

Task 3: Months 24-48: Creation and testing of mutations of p21 with different affinities for cyclins D1 and E.
Ongoing.

Task 4: Months 24-48: Creation and testing of versions of p21 with variation of the distance between the cyclin-binding and the cdk2 binding sites (in cis).

Technical objective 2. Analysis of the interaction between p21, PCNA and Fen1.

Task 5: Months 1-12: Determination of which part of PCNA interacts with p21 and with Fen1.
Completed.

Tasks 6 and 7: Months 24-48: Designing D amino acid based retro-inverse peptides that mimic the PCNA interacting portion of p21. Analysis of their ability to inhibit PCNA in vitro.
Ongoing.

Task 8: Months 36-48: Testing effect of adding Fen1 to replication reactions or over-expressing Fen1 in MCF-7 cells in culture.

METHODS.

All methods have been described in the annual report of '99.

RESULTS AND DISCUSSION

Our effort this year has been focused on repeating all the assays as quantitatively as possible to obtain reliable data suitable for publication. One manuscript reporting these results has been submitted for publication and another is in preparation.

Purification of Enzymes and Substrates. To determine the contribution of the Cy motif to a cyclin/cdk substrate, we constructed a series of recombinant peptide substrates derived from the replication factor, HsCDC6. These peptides all contain a cyclin/cdk phosphorylation site at the N-terminus and either a wild-type Cy motif (CDC6(wt)), a mutated Cy motif (CDC6(mut)), or a null Cy motif (CDC6(null))(Fig 1). We postulated

that these peptides would be ideal substrates for this study considering that (1) the N-terminal SPXK is known to be phosphorylated by cyclin/cdk complexes *in vitro* and (2) the phosphorylation of this site *in vivo* is dependent upon an intact Cy motif. The two sites are in close proximity in the amino acid sequence of HsCDC6 (~ 20 residues) allowing a peptide to easily span this region. After expression of these peptides in *E. coli*, they were purified to homogeneity before their use in the kinetic studies (data not shown). Cyclin A/cdk2 and cyclin E/cdk2 were also purified to homogeneity as determined by SDS-PAGE and Coomassie Blue staining (Fig. 2). The identities of the proteins were confirmed by western blotting with the appropriate antibodies (data not shown). Phosphorylation of cyclin A/cdk2 and cyclin E/cdk2 with bacterially expressed CIV1 resulted in a 2-fold increase in velocity suggesting that the purified cyclin/cdk complexes were not completely phosphorylated on Thr160.

Determination of Kinetic Parameters. Using purified enzyme and the peptide substrates, we developed a highly reproducible kinase assay. Phosphorylation of the peptide substrates by both cyclin A/cdk2 and cyclin E/cdk2 followed hyperbolic kinetics and increased linearly as a function of both enzyme concentration and time when substrate concentrations were not limiting (data not shown). All further experiments were carried out using conditions within this linear range to ensure the results could be interpreted using Michaelis – Menten based equations.

Initial velocities were determined for both cyclin A/cdk2 and cyclin E/cdk2 complexes using our CDC6-based peptides as substrates. These velocities were plotted against ATP concentrations on a double-reciprocal plot using various fixed concentrations of peptide substrate. A representative plot in which cyclin E/cdk2 was used to phosphorylate the CDC6(wt) peptide is shown in (Fig 3A). In all of these plots, the intersecting pattern of initial velocities is consistent with a sequential kinetic mechanism in which both substrates (ATP and peptide) must be bound before any products are released. From this data, however, we are unable to show whether substrate addition is an ordered or random process. Kcat and Km for a given substrate/enzyme pair were determined by secondary plots of the slopes and intercepts of the initial velocity lines versus reciprocal substrate concentration (Fig 3, B and C). A summary of the data for all of the enzymes and substrates can be found in Table 1.

The wild-type substrate was efficiently bound by both cyclin A/cdk2 and cyclin E/cdk2 as demonstrated by Km values of 1.7 μM and 7.9 μM, respectively.
Upon mutation of the Cy motif in the N-terminus from RRLVF to RAARA, these values increased 75-fold to 145 μM for cyclin A/cdk2 and 120-fold to 970 μM for cyclin E/cdk2. These dramatic increases in Km demonstrate the importance of the Cy motif in targeting substrates to these enzyme complexes. The Km values for CDC6(mut) were 27 μM and 165 μM for cyclin A/cdk2 and cyclin E/cdk2, respectively, a 15-fold and 20-fold increase compared to the wild-type peptide. Thus, this mutation produces a partially functional Cy motif, rather than a completely non-functional motif.

In contrast to the Km values for the peptide substrates, The Kcat and the Km^{ATP} values for the enzymes remained very similar with less than a 4-fold change between substrates. This would suggest that although the Cy motif plays a critical role in increasing the affinity of cyclin/cdk complexes for a particular substrate, it does not significantly increase the efficiency of phosphoryl transfer from ATP to the peptide.

Competition with Cy-motif containing Peptides from p21. To further demonstrate that the Cy motif acts as a docking site for the interaction of substrate with enzyme, we tested the ability of a Cy-motif containing peptide, PS100, derived from p21 to inhibit the phosphorylation of our peptide substrates. If the Cy motif truly directs substrates in this manner, then the PS100 peptide is expected to inhibit the phosphorylation of Cy motif-containing substrates such as our CDC6(wt) and CDC6(mut) peptides but unable to inhibit

CDC6(null) which lacks a Cy motif. The data are shown in Figures 4A and 4B. The concentration of the substrate peptides had to be adjusted to obtain equivalent phosphorylation by cyclin/cdk complexes, with more of CDC6(null) being used relative to CDC6(wt) or CDC6(mut). Despite this, a comparison of the ratio of the inhibitor to substrate for any given peptide substrate shows that PS100 selectively inhibits the phosphorylation of only Cy motif containing substrates, CDC6(wt) and CDC6(mut), but not that of CDC6(null). DTM101, a peptide containing a scrambled Cy motif, does not inhibit the phosphorylation of any of the substrates (data not shown), consistent with our previous results that a negative control inhibitory peptide containing a mutation in the Cy motif does not inhibit the phosphorylation of Rb (4).

Considering the unusual shape for the inhibition curve of cyclin A/cdk2 with the CDC6(wt) peptide and PS100, we carried out a systematic inhibition study to determine the mode of inhibition of PS100 for both cyclin A/cdk2 and cyclin E/cdk2 using the CDC6(wt) peptide as the substrate. Lineweaver-Burke plots for these inhibition experiments are shown in Figure 5A and 5B for cyclin E/cdk2 and cyclin A/cdk2, respectively. Visual inspection of these plots shows that PS100 competitively inhibits the phosphorylation of the CDC6(wt) peptide by both cyclin E/cdk2 and cyclin A/cdk2. From these data, we were also able to determine the inhibition constant (Ki) for PS100 which was 7.5 ± 0.5 for cyclin E/cdk2 and 116.3 ± 11.8 for cyclin A/cdk2.

Effects of Linker Length on Substrate Phosphorylation. The Cy motif and the cdk phosphorylation site must be simultaneously engaged with the cyclin/cdk complex or the Cy motif might first bind to the cyclin in order to increase the local concentration of the substrate around the enzyme and is then released to allow the phosphoacceptor serine to bind the kinase active site. To distinguish between these two possiblities, we reasoned that simultaneous engagement of both binding sites would require the Cy motif and the phosphorylation site to be separated by an amino acid linker of sufficient length to span the 40A° distance from the binding site on the surface of the cyclin to the catalytic site on the cdk. The bind and release mechanism, on the other hand, would be independent of the length of the amino acid linker. To test this hypothesis, we systematically replaced the wild-type amino acid linker (16 residues) connecting the cdk phosphorylation site and Cy motif of our CDC6 peptide with flexible predominantly polyglycine linkers of 2, 6, 12, or 18 residues. Assuming the flexible linkers would extend on the average 4A°/residue, the distance separating the two sites on these substrate peptides would be 8, 24, 48, and 72 A°, respectively. These substrates were made in the context of both the CDC6(wt) and CDC6(null) peptides and then tested for their ability to be phosphorylated by cyclin A/cdk2 and cyclin E/cdk2. By comparing the phosphorylation of the wild-type versus the null peptides, we were able to specifically determine the contribution of the Cy motif for a given linker length and thus eliminate any artifacts that may arise from differential binding of the shorter peptides to p81 phosphocellulose. As shown in Fig. 6A and 6B, we found that only substrates containing both an intact Cy motif and either the 12 or the 18 residue linker were effectively phosphorylated. Substrates that either lacked a Cy motif or contained a linker that was unable to span the distance from the cdk binding site to the cyclin binding site were phosphorylated extremely poorly. This length dependence of the linker strongly suggests that both the Cy motif and the cdk phosphorylation site must be simultaneously bound to cyclin/cdk complex to promote its efficient phosphorylation and eliminates the bind and release model of substrate phosphorylation.

Mutagenesis of Cy motif of p21N. As an alternative method of defining what exactly is a functional Cy motif we created a library of mutations in the Cy motif of p21N in the second year of this project. In this year we expressed and purified each of the GST-p21N derivatives and assayed them for their inhibitory potency on the phosphorylation of the CDC6 peptide substrate by cyclin E/cdk2 and cyclin A/cdk2. (Table 2). This data is

significantly different from the preliminary data we reported in last year's annual report. Because of a technical error in the first set of measurements we were not in conditions of initial velocity. We have, therefore discarded those results. The current results, however, have been verified with 2-3 independent preparation of p21N and enzymes at different times through the year.

KEY RESEARCH ACCOMPLISHMENTS.

- Discovered that contrary to current view the phosphoacceptor serine (in the context of SPXK) by itself is an inefficient substrate of cyclin dependent kinases.
- The addition of a cyclin binding motif (Cy motif) at a defined distance from the phosphoacceptor serine is essential for making an optimal substrate (with a Km in the few μM range).
- Cy motif based cdk inhibitors may be specific inhibitors of cyclin E-cdk2, a matter of some importance to the 20-30% breast cancers that selectively up-regulate cyclin E levels.
- Contrary to current view an RXL is neither necessary nor sufficient to be an effective Cy motif. The exact requirement seems to be for a hydrophobic patch, suggesting that Cy-mimetic chemicals need to be hydrophobic.

REPORTABLE OUTCOMES.

Manuscripts

under review in Oct. 2000:
Takeda D, Wohlschlegel J, Dutta A. A bipartite substrate recognition motif for cyclin-dependent kinases. J. Biological Chemistry

in preparation in Oct. 2000:
Wohlschlegel J. Dwyer BT, Takeda D, Dutta A. A mutational analysis of the Cy motif from p21 reveals sequence degeneracy and specificity for different cyclin-dependent kinases.

Abstracts

Dutta A. Keystone meeting on the Cell Cycle. Jan. 8-Jan. 13, 2000. Colorado.

Dutta A. American Association of Cancer Research, annual meeting, April 1- April 5, 2000. San Francisco.

Dutta A. Era of Hope meeting, U.S. Army Medical Research, Breast Cancer Program. June 8-June 12, 2000. Atlanta.

Dutta A. Gordon Conference on Molecular Genetics of Cell Proliferation. July 22- July 27, 2000, New Hampshire.

Wohlschlegel J. Cell cycle meeting, May 17-21, 2000. Cold Spring Harbor Laboratory, New York.

Presentations that included work supported by this award

Dutta A.

International meeting on "Role of protein phosphorylation in signal transduction", Osaka Bioscience Institute, Osaka, Japan. May 10-12.
University of Colorado Health Sciences Center, Denver, Jan 13
Stanford University School of Medicine, April 6
Seoul National University, Seoul, S. Korea, May 4
Samsung Biomedical Research Institute, Seoul, S. Korea, May 4
Nagoya City University, School of Medicine, Nagoya, Japan, May 8
Nagoya University School of Medicine, Nagoya, Japan, May 8
Nara Institute of Biomedical Science, Nara, Japan, May 9
Osaka University, Dept. of Biology, Osaka, Japan, May 10
Tokyo University Institute of Medical Sciences, Tokyo, Japan, May 15
Vanderbilt University, Oct. 12

Funding applied for based on work supported by this award

RO1 from the National Institutes of Health: "Role of Cy motifs in substrate recognition by CDKs". P.I. Anindya Dutta.

Predoctoral Fellowhsip from the U.S. Army's breast cancer program: "Discovery and development of inhibitors that selectively interfere with cyclin-dependent kinase substrate recognition." P.I. James Wohlschlegel.

Predoctoral Fellowship from the Howard Hughes Medical Institute: P.I. Mr. David Takeda.

Employment applied for based on work supported by this award

Senior research assistant, Genetics Institute and Millennium Pharmaceuticals. Mr. Brian Dwyer.

Ph.D. program in Biological and Biomedical Sciences of Harvard Medical School. Mr. David Takeda.

CONCLUSIONS

The Cy motif is part of a bipartite substrate recognition sequence for cdks. We have used a series of peptide substrates derived from HsCDC6 to determine the contribution of a Cy motif to the phosphorylation of a substrate by cyclin/cdk complexes. This detailed kinetic analysis of the phosphorylation of these substrates reveals its importance in substrate recognition by cyclin/cdks and provides additional insight into its mechanism of action.

The CDC6 wild type peptide was efficiently phosphorylated *in vitro* by both cyclin E/cdk2 and cyclin A/cdk2 complexes. The measured Km for the peptide was less than 10 μM for both enzymes suggesting the existence of a high affinity interaction between the enzyme and our substrate. This is in contrast to previously characterized substrates whose Km values were no lower than 200 μM – 100-fold greater than our peptide (18). Since these previously characterized substrates contained only the consensus S/T-P-X-K/R phosphorylation site, this reduction in Km for our peptides can likely be attributed to the presence of a Cy motif. Indeed, the presence of this Cy motif makes the wild-type CDC6 peptide the most efficient peptide substrate of cyclin/cdk complexes characterized to date.

The extremely efficient phosphorylation of our CDC6(wt) peptide is surprising considering a study by Solomon *et al.* which defined the sequence requirements of the consensus cdk phosphorylation site (18). They showed that a SPPK phosphorylation site, like that present in CDC6, is phosphorylated at less than 5% of the level of the SPRK phosphorylation site of their wild-type peptide. This decrease in phosphorylation can be attributed to the enzyme's inability to tolerate a proline at the third position of the sequence. Their result is consistent with our data for the CDC6(null) peptide which is poorly phosphorylated by cyclin/cdk complexes. Hence, we conclude that the addition of a Cy motif is sufficient to convert a peptide whose phosphorylation site would normally make it a poor substrate into a very efficient substrate, emphasizing the contribution of the Cy motif to the enzyme-substrate interaction. Therefore, substrate recognition by cyclin/cdks occurs through a bipartite recognition sequence on the substrate consisting of both the cdk phosphorylation site (S/T-P-X-K/R) and the cyclin binding Cy motif.

By studying the effects of linker length on substrate phosphorylation, we have shown that both the Cy motif and the cdk phosphorylation site must be simultaneously bound to the cyclin/cdk complex. Previous work suggests that the purpose of the Cy motif was to increase the local concentration of the substrate around the enzyme (20). Our results suggest that in addition to this role, the Cy motif may also be responsible for orientating specific cdk phosphorylation sites with respect to the active site of cdk2 to further facilitate their phosphorylation – a mechanism that requires the concurrent binding of the Cy motif and cdk phosphorylation site to the enzyme as seen with the CDC6-derived substrates. For example, binding of the Cy motif of a substrate to the cyclin might conformationally restrain the substrate such that only particular cdk phosphorylation sites are accessible to the cdk. In this way, the Cy motif would not only increase the overall affinity of the cyclin/cdk for the substrate, it would also specify which phosphorylation sites would be targeted by the kinase.

A peptide containing the Cy motif of p21 specifically inhibits substrates with Cy motifs: We had earlier reported that the Cy motif of p21 inhibited the phosphorylation of pRb but not histone H1 (4). Now we show that a Cy motif containing peptide (PS100) is able to selectively inhibit only Cy motif containing substrates. This is consistent with PS100 competing with substrate for the binding site on the cyclin and confirms our model in which the Cy motif targets substrates to the enzyme via a docking site on the cyclin. If the physiological targets of cyclin-cdks necessarily use the Cy-cyclin interaction, peptides or chemicals that mimic the Cy motif are likely to be specific inhibitors of cdks and will differ from existing inhibitors that target the ATP binding site. Indeed, preliminary studies show that such peptides lead to the selective killing of only transformed cells in which the E2F pathway has been deregulated (19). We al;so show that the Cy motif based inhibitors are classic competitive inhibitors of cdk activity.

Specificity of cyclins for substrates may be determined by the Cy motif. Not much is known about how the specificity of cyclin /cdk complexes is determined. Our results suggest one mechanism by which this specificity could be achieved. The Km for CDC6(wt) was 1.7 μM for cyclin A/cdk2 and 7.9 μM for cyclin E/cdk2 suggesting that both enzymes have a high affinity for the Cy motif present in this particular peptide. In contrast, CDC6(mut) had a Km of 27 μM for cyclin A/cdk2 but 163 μM for cyclin E/cdk2. Therefore, cyclin A/cdk2 but not cyclin E/cdk2 could effectively phosphorylate the mutant substrate. Thus, although the wild-type Cy motif interacted strongly with both enzymes, mutations could be made in the Cy motif which confer specificity to cyclin A/cdk2 over cyclin E/cdk2. We also observed that the inhibitory PS100 peptide containing the RRLFG Cy motif was a far better inhibitor of cyclin E/cdk2 (Ki = 7.5 μM) than cyclin A/cdk2 (Ki = 116.3 μM). Based on these results, it seems likely that different Cy motifs will

preferentially associate with a specific cyclin/cdk complex and thereby target that substrate for phosphorylation by only that enzyme.

Mutagenesis of Cy motif on p21N: Based on the mutations in p21N we conclude that it should be possible to design chemicals that look like the Cy motif and inhibit cyclin-cdk.

Deletion of the Cy motif of p21N provided a surprise. This derivative could no longer inhibit cyclin E/cdk2 consistent with the notion that a Cy motif peptide inhibits the kinase. In contrast, deletion of the Cy motif did not have any effect on the inhibition of cyclin A/cdk2. This result is in stark contrast to the crystal structure of p27N complexed with cyclin A/cdk2 which clearly showed that the Cy motif interacts with the hydrophobic patch of cyclin A. In addition, Cy motifs decrease the Km of a substrate for cyclin A/cdk2 (Table I) and a Cy peptide inhibits the phosphorylation of a substrate by cyclin A/cdk2(Fig. 5) . Therefore Cy motifs in other contexts clearly interact with the hydrophobic patch of cyclin A. The likely explanation is that p21N has another sequence that docks on cyclin A so that the Cy motif is not absolutely essential. We conclude, therefore that it will be possible to design Cy mimetic chemicals that are more specific for cyclin E/cdk2 than cyclin A/cdk2. If we can discover whether p21NDCy has another site that interacts with cyclin A (but not cyclin E) then we can also propose the design of chemicals that inhibit cyclin A/cdk2 better than cyclin E/cdk2.

Intriguingly, RXL is not sufficient to act as a Cy motif on cyclin E/cdk2. This conclusion is based on the inability of RRLAA and RALAG to inhibit the kinase. Therefore a Cy mimetic chemical has to be larger than an RXL tripeptide. Most likely it has to be flanked by hydrophobic residues.

Another point of interest is that R1 can be successfully replaced by L or V (LRLFG or VRLFG) and L3 can be replaced by V (RRVFG) to produce a functional Cy motif. The crystal structure of p27 complexed with cyclin A/cdk2 had indicated that the aliphatic chain of R1 executes a hydrophobic interaction with the hydrophobic patch on the cyclin and that the positive charge on the epsilon amino group was irrelevant for this interaction. The fact that a polar residue (L or V) can substitute for R1 supports this conclusion.

Overall, these results emphasize that the ideal Cy mimetic chemical will most likely be a hydrophobic chemical extended over a distance of at least 3-4 peptide bonds. Such a chemical will be a potent competitive inhibitor of cyclin-dependent kinases, might show specificity against cyclin E/cdk2 over cyclin A/cdk2 and be effective for treating subsets of breast cancers that over-express cyclin E in the malignant epithelial cells.

.FIGURE LEGENDS

Figure Legends

Fig. 1. Schematic of Substrate Peptides Derived from HsCDC6. Peptides were constructed that spanned the consensus cdk phosphorylation site from residues 74-77 and the Cy motif from residues 94-98. The N-terminal cdk phosphorylation site and the C-terminal Cy motif are highlighted in bold.

Fig. 2. SDS-PAGE of cyclin E/cdk2 and cyclin A/cdk2. Purified Cyclin E/Cdk2 (lane 1) and Cyclin A/Cdk2 (lane 2) were loaded on a 12% gel and the proteins were stained with Coomassie Blue.

Fig. 3. Representative Initial Velocity Patterns and Secondary Plots. (A) Initial velocity pattern was determined for cyclin E/cdk2 using ATP as the varied substrate and the following fixed concentrations of CDC6 wild-type peptide: 2.5 μM (●), 5 μM (X), 10 μM (□), 20 μM (■), and 40 μM (♦). (B) Secondary plot of primary slopes vs. reciprocal

peptide concentration. (C) Secondary plot of primary intercepts vs. reciprocal peptide concentration.

Fig. 4. Cy-motif containing peptide selectively inhibits the phosphorylation of only Cy-motif containing substrates by (A) cyclin E/cdk2 and (B) cyclin A/cdk2. The Cy Motif Containing peptide PS100 is able to inhibit the phosphorylation of 5 μM CDC6(wt) (●), 50 μM CDC6(mut) (o) but not 1 mM of CDC6(null) (♦) substrate.

Fig. 5. PS100 competitively inhibits the phosphorylation of the CDC6(wt) substrate by (A) cyclin E/cdk2 and (B) cyclin A/cdk2. Initial velocities were determined in the presence of different fixed concentrations of the PS100 peptide: 0 μM (♦) , 6.25 μM (o), 12.5 μM (□), and 25 μM (●) for cyclin E/cdk2 and 0 μM (♦), 100 μM (o), 200 μM (□) and 500 μM (●) for cyclin A/cdk2.

Fig 6. Phosphorylation of peptide sustrates by (A) cyclin E/cdk2 and (B) cyclin A/cdk2 is dependent on the length of the linker connecting the N-terminal cdk phosphorylation site and the C-terminal Cy motif. For each given linker length, the velocites were determined for both the wild-type Cy motif (▒) and the null Cy motif (■).

Figure 1

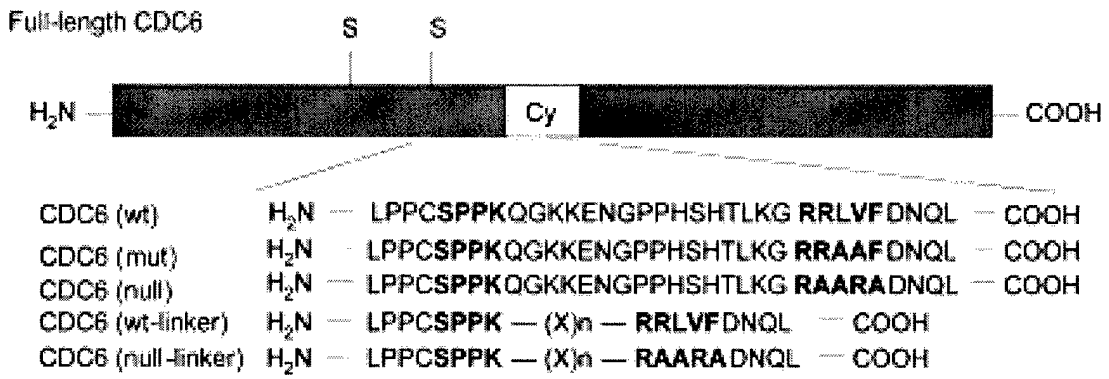


Figure 2

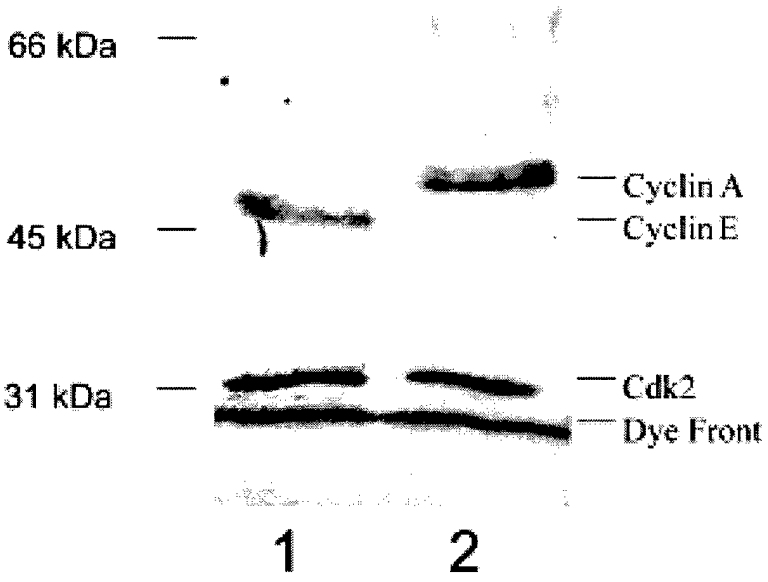
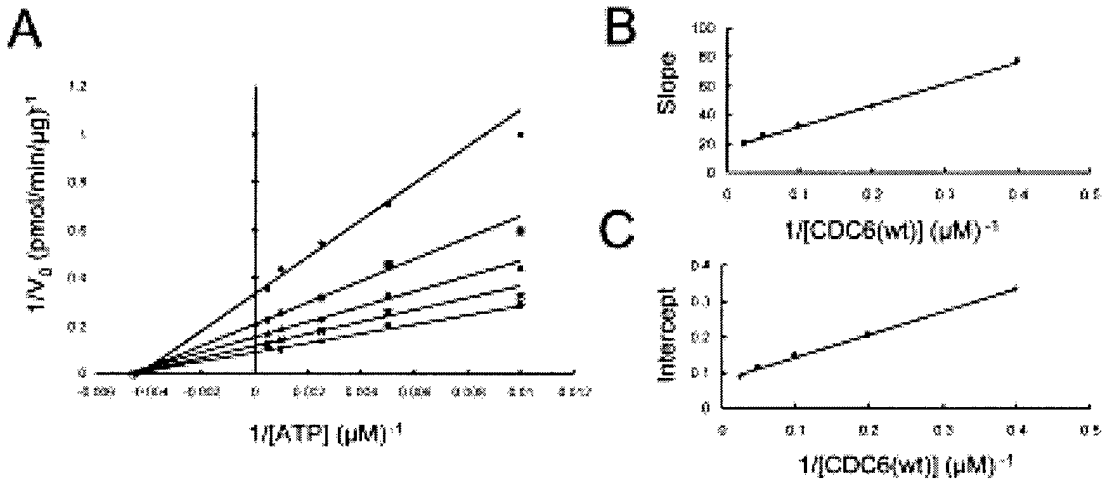


Figure 3



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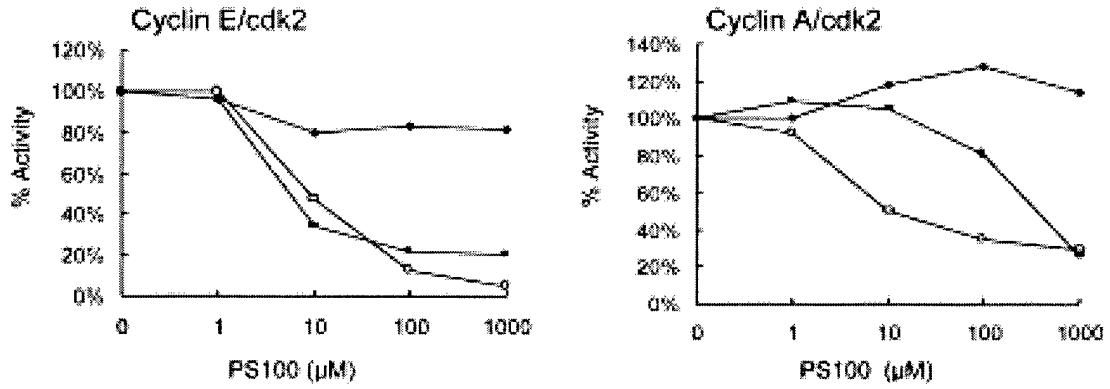

Figure 5

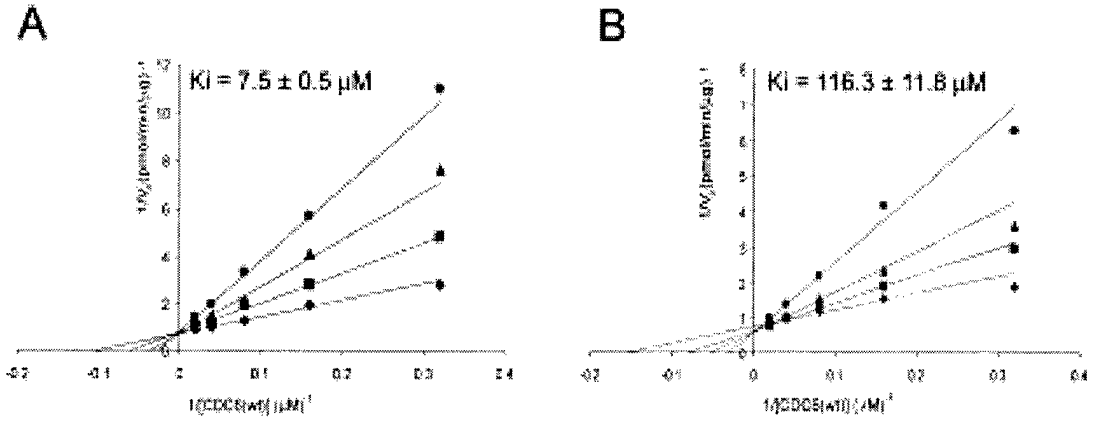

Figure 6

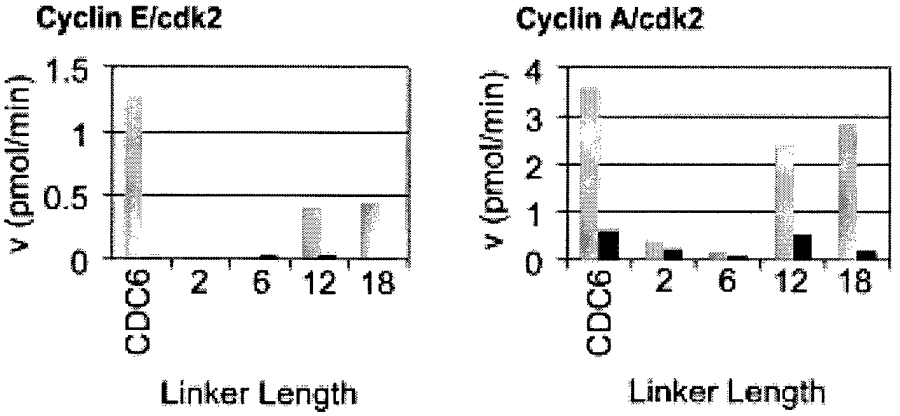

TABLE 1

Table 1. Kinetic Parameters for cyclin/cdk complexes and CDC6-derived peptides. Units for K_m^{ATP} and $K_m^{peptide}$ are expressed in mM while units for k_{cat} are in min^{-1}.

		CDC6 (wt)	CDC6 (mut)	CDC6(null)
CyclinE/cdk2	k_{cat}	86.6 ± 5.0	60.1 ± 11.1	25.0 ± 4.0
	$K_m^{peptide}$	7.9 ± 0.53	163 ± 34	970 ± 184
	K_m^{ATP}	206 ± 16	165 ± 32	377 ± 18
CyclinA/cdk2	k_{cat}	0.82 ± 0.06	2.57 ± 0.16	0.62 ± 0.09
	$K_m^{peptide}$	1.7 ± 0.33	27 ± 2.4	145 ± 34
	K_m^{ATP}	34 ± 13	84 ± 16	103 ± 16

TABLE 2

The IC50 (nM of inhibitor at which kinase activity of indicated cyclin-cdk is inhibited by 50%) of p21N wild type and its derivatives at the Cy motif. Nomenclature: R1A indicates that the first arginine of the RRLFG motif has been changed to alanine.

Inhibitor	Cyclin E/cdk2	Cyclin A/cdk2
p21N (RRLFG)10	100	100
ΔCy	1000	100
R1A	1000	100
R2A	100	100
L3A	1000	250
F4A	100	100
G5A	100	100
R1L	100	100
R1V	100	100
L3V	100	100
p21N (RRLAA)	1000	100
p21N (RALAG)	1000	100

REFERENCES

1. Morgan, D. O. (1995) *Nature* **374**(6518), 131-4
2. Morgan, D. O. (1997) *Annu Rev Cell Dev Biol* **13**, 261-91
3. Adams, P. D., Sellers, W. R., Sharma, S. K., Wu, A. D., Nalin, C. M., and Kaelin, W. G., Jr. (1996) *Mol Cell Biol* **16**(12), 6623-33
4. Chen, J., Saha, P., Kornbluth, S., Dynlacht, B. D., and Dutta, A. (1996) *Mol Cell Biol* **16**(9), 4673-82
5. Zhu, L., Harlow, E., and Dynlacht, B. D. (1995) *Genes Dev* **9**(14), 1740-52
6. Saha, P., Eichbaum, Q., Silberman, E. D., Mayer, B. J., and Dutta, A. (1997) *Mol Cell Biol* **17**(8), 4338-45
7. Saha, P., Chen, J., Thome, K. C., Lawlis, S. J., Hou, Z. H., Hendricks, M., Parvin, J. D., and Dutta, A. (1998) *Mol Cell Biol* **18**(5), 2758-67
8. Adams, P. D., Li, X., Sellers, W. R., Baker, K. B., Leng, X., Harper, J. W., Taya, Y., and Kaelin, W. G., Jr. (1999) *Mol Cell Biol* **19**(2), 1068-80
9. Russo, A. A., Jeffrey, P. D., Patten, A. K., Massague, J., and Pavletich, N. P. (1996) *Nature* **382**(6589), 325-31
10. Dutta, A., and Bell, S. P. (1997) *Annu Rev Cell Dev Biol* **13**, 293-332
11. Williams, R. S., Shohet, R. V., and Stillman, B. (1997) *Proc Natl Acad Sci U S A* **94**(1), 142-7
12. Petersen, B. O., Lukas, J., Sorensen, C. S., Bartek, J., and Helin, K. (1999) *Embo J* **18**(2), 396-410
13. Jiang, W., Wells, N. J., and Hunter, T. (1999) *Proc Natl Acad Sci U S A* **96**(11), 6193-8
14. Delmolino, L., and Dutta, A.
15. Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L. H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., and et al. (1995) *Mol Biol Cell* **6**(4), 387-400
16. Blacklow, S. C., and Kim, P. S. (1996) *Nat Struct Biol* **3**(9), 758-62
17. Endicott, J. A., Noble, M. E., and Tucker, J. A. (1999) *Curr Opin Struct Biol* **9**(6), 738-44
18. Holmes, J. K., and Solomon, M. J. (1996) *J Biol Chem* **271**(41), 25240-6
19. Chen, Y. N., Sharma, S. K., Ramsey, T. M., Jiang, L., Martin, M. S., Baker, K., Adams, P. D., Bair, K. W., and Kaelin, W. G., Jr. (1999) *Proc Natl Acad Sci U S A* **96**(8), 4325-9
20. Schulman, B. A., Lindstrom, D. L., and Harlow, E. (1998) *Proc Natl Acad Sci U S A* **95**(18), 10453-8